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WITNESS my hand this
Tenth day of December 2003

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Patents Act 1990

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PROVISIONAL SPECIFICATION

Invention Title:

Restoration of Methylation States in Cells

The invention is described in the following statement:

Technical Field

The invention relates to methods to restore or alter DNA methylation patterns in cells, particularly in cells having methylation patterns which may have been corrupted or altered.

Background Art

The complete information necessary to encode the structure of all gene products of an organism such as an animal (or a plant) is stored in the sequence of the four deoxynucleotides adenine (A), guanine (G), thymine (T) or cytosine (C) in its deoxyribonucleic acid (DNA). There is, however, a fifth deoxynucleotide in DNA produced as a result of the post -replication methylation of some of the C deoxynucleotides (mC). One of the functions of the mC is to act as a developmental signal determining whether or not a particular gene is active and able to be transcribed in order for its gene product to be made. The methylated state signals silencing of a gene and the unmethylated state signals activation of a gene of cells of different tissue types. Methylation can change in a coordinated fashion according to a genetically controlled pattern at various stages in the development of a whole adult from a fertilised egg. The precise way in which this happens, its causes and how these processes are controlled are yet to be discovered.

Methylation patterns in adult differentiated cells of different cell types differ each from each other. Normally these different patterns of methylation are quite stable through many cell divisions but under certain circumstances they can be modified. For example, methylation is thought to be part of the process involved in the cloning of animals, where the nucleus from an adult fully differentiated cell (such as an epithelial cell) is inserted in the cytoplasm of a de-nucleated embryonic stem cell. The cloned cell is reprogrammed so that it takes on the developmental potential of the nucleus which was removed form the cell. Whilst the detailed evidence is slight, this process is thought to involve the reprogramming of the methylation pattern of the genome of the transplanted nucleus.

Unprogrammed modification of the normal methylation pattern in the genome often is deleterious to the health of the individual, and in humans, can lead to life-threatening disease such as cancer. This has lead to the clinical usage of some methods which are known to modify methylation of cells in tissue culture, such as treating the cells with 5-aza cytidine which inhibits the function of the methylating

enzyme 5-methyl transferase and after further rounds of DNA replication, leads to global de-methylation of many genes in the genome. Whilst it has had limited success in treating certain types of cancer, it also has toxic side effects, as might be expected.

So far it has not proven possible to selectively de-methylate or methylate specific C in the genome in living cells. The goal of reprogramming methylation states to restore the corrupted methylation patterns of aged or diseased cells in a coordinated fashion to date has only been a dream.

As individual cells age or are exposed to environmental insults of various sorts their genomes also may be damaged. Normally, such damage is repaired in some way or another. Errors occur in the repair processes which lead to changes in the genetic code in the cell's DNA and thus to mutations if the corruption is limited to a change in one or other of the four coding nucleotides A, G, T or C. If, however, the corruption involves a change in the pattern of 5' methyl cytosine (5mC) nucleotides in the controlling elements of genes, it can result in genes being activated if a particular 5mC is replaced by a C or silenced if a C is replaced by a 5mC. DNA-damaging agents such as certain drugs or ionising radiation can produce such a corruption of the methyl cytosine (mC) pattern in genes. Moreover, with the passage of time, cells accumulate similar changes in their DNA which have the effect of modifying in a deleterious fashion normal cellular function resulting in increasing levels of disability of the aging individual or may result in the induction of a disease such as some types of cancer in the individual having such damaged cells.

The present inventors has devised a means of reprogramming the mC pattern in cells to overcome or alter the deleterious effects of other changes in the mC patterns in cells.

Disclosure of Invention

In a first aspect, the present invention provides a method for altering the methylation state of a cell comprising:

(a) treating a target cell having an undesired methylation pattern in its genome DNA so as to make the cell permeable to macromolecules;

(b) obtaining a cell free extract from a donor cell having a desired methylation pattern in its genome DNA; and

(c) exposing the treated cell to the cell-free extract from the donor cell under suitable conditions and period of time to obtained a modified cell having at least a part of its methylation pattern altered to conform with the corresponding methylation pattern of the donor cell.

The method may further include:

(d) culturing the modified cell to obtain multiple copies of the modified cell.

Preferably, the target cell is a cell derived from an individual suffering from age-related disabilities, or from a disease such as cancer. More preferably, the target cell is a stem cell. It will be appreciated that other cell types, particularly diseased cell types could also be treated.

Preferably, the donor cell is a cell derived from a normal or healthy individual of a cell type similar to cell type having the damaged genome More preferably, the donor cell is a stem cell. The donor cell can be from a juvenile or subject not suffering from a condition or state caused by the undesired methylation state of the target cell. It will be appreciated that the other cell types such as bone marrow stem cells, resultant stem cells, osteoliant stem cells, and epithelial cells could also be used as donor cells.

Preferably, the target cell and the donor cell are of the same cell type.

By stem cells, it is meant to include all adult stem cells and particularly those of bone marrow lineages.

The target cell can be treated by any suitable means to render it permeable to the passage of macromolecules. Treatment includes, but is not limited to, electroporation, low temperature thermal shock, various enzymes such as streptolysin O. More preferably, the treatment renders the cell temporally permeable.

The cell-free extract can be obtained by any suitable means known to the art. Examples include, but not limited to, that described by Hakelien et al (2002) Nature Biotechnology 20:460-466. It will be appreciated that the cell-free extract may be further processed or fractionated to obtain components or macromolecules from the donor cell that, when in a cell, will alter the methylation pattern of the cell.

The exposure time can from minutes to hours, depending on the cell type, cell-free extract and the conditions of treatment. The treatment may be done at physiological temperature, or any other temperature which will not result in the death of the cell.

The modified cell may be cultured in any suitable media known to the art under conditions that are suitable for cell growth and division. The present invention results in the production of stable modified cells having a desired methylation pattern in their genome DNA.

In a preferred form, the DNA methylation pattern is restored to 'normal' juvenile pattern in the whole of the genome. It will also be appreciated that methylation of only a part of the genome DNA can also occur.

The DNA methylation pattern characteristic of one cell type may be changed to that characteristic of one subtype of that of another subtype. For example, from one lymphocyte T cell subtype to another cell type.

The method may result in the reprogramming of a corrupted methylation pattern of a single cell type to that of the pattern of a corresponding normal cell of the same type.

The method may be used to reprogram the methylation pattern of old cells to that of young cells. For example, the methylation pattern of old adult stem cells can be reprogrammed to a pattern of young stem cells. The methylation pattern of the genome of diseased stem cells can be altered to that of normal stem cells.

The stem cells can be whose cells with DNA that has been damaged by exposure to drugs or damaging electromagnetic radiation, for example.

In a second aspect, the present invention provides a modified cell obtained from the method according to the first aspect of the present invention.

Preferably, the modified cell is a stem cell. The modified cells can be used as donor cells for subsequent treatments which would obviate the need to obtain fresh donor cells from individuals each time a treatment is carried out. The modified cells may also be useful as cells lines for research or other medical uses.

In a third aspect, the present invention provides a method for treating an individual suffering from a condition resulting from having cells with an undesired methylation pattern in their genome, the method comprising:

(a) obtaining cells from the individual;

(b) carrying out the method according to the first aspect of the present invention on the cells to obtain modified cells having a desired methylation state or pattern; and

(c) returning the modified cells to the individual where the cells multiply and replace at least some cells having the undesired methylation pattern so as to treat the condition.

Preferably, the individual is suffering from a condition such as disabilities associated with aging or with cancer.

Preferably, the cells are stem cells. The modified stem cells can then differentiate in the subject to assist in the treatment. It will be appreciated that the other cell types such as cancer cells or those associated with another disease could also be treated.

The present invention is particularly useful to restore methylation patterns in dysfunctional cells having methylation patterns which have been corrupted as a consequence of age-related processes, by exposure to drugs or to more random events.

An advantage of the present invention is that the individual's cells, after reprogramming are returned to the subject. Accordingly, there should not be any problems of rejection or the requirement to use immunosuppressive drugs or medication.

The method can be used for individual or personalised treatment, depending on the subject and condition.

Cells which have been made temporarily permeable to macromolecules and were treated with a cell-free extract of normal cells of the same phenotype. Such cells may be derived from the treated host individual or more preferably from a juvenile who has not been exposed to the same environmental insults (such as age) which caused the problem.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this application.

In order that the present invention may be more clearly understood, preferred forms will be described with reference to the following examples.

Mode(s) for Carrying Out the Invention

EXAMPLE

Blood-derived stem cells

Take blood-derived bone marrow stem cells from an aged donor or a donor who has been treated with DNA damaging agents. These stem cells have been shown to have a corrupted methyl cytosine (MC) pattern in their genomes. Expose the cells for a period (of a few minutes to many hours) to a cell-free extract obtained from a sample of stem cells from a young healthy donor then transfer the exposed cells to fresh culture medium and incubate them for a period before transferring the treated stem cells back into the host with the damaged stem cells.

METHODS AND MATERIALS

Preparation of cell extracts

A suitable protocol is set out below.

(i) $2x10^6$ purified CD34+ cells washed and resuspended in 100 µl of PBS or suitable culture medium then frozen in liquid nitrogen for storage.

(ii) The cells were thawed then washed in a suitable ice-cold lysis buffer then centrifuged at 1500 rpm for 10 minutes to sediment the cells in a pellet.

(iii) The cell pellets were then resuspended in 200 µl of lysis buffer.

(iv) Cells and nuclei were then disrupted using a 2 mm diameter sonicator to form a lysate.

(v) The lysate was cleared by centrifugation at 15,000g for 15 minutes at 4°C.

(vi) Cell extracts were used either freshly prepared or could be stored at –70°C without noticeable loss of activity.

Cell reprogramming

(i) Cells to be reprogrammed were grown on round 4 cm 6 well nuclon tissue culture dished in a suitable medium for the desired cell type until 25-75% confluence had been reached.

(ii) Cells were permeabilised by the addition of 200 ng/ml streptolysin O in Ca^{2+} free culture medium for 30-60 minutes at 37°C.

(iii) The culture medium was then aspirated off and the cells then overlayed with the CD34+ cell extracts containing 1 mM ATP, 10 mM creatine phosphate, 25 µg/ml creatine kinase and 100 µM GTP and incubated at 37°C for 1-4 hours.

(iv) The cell membranes were then 'resealed' by the addition of culture medium containing 2 mM $CaCl_2$ and the cells incubated at 37°C for 1-4 hours.

(v) Finally, the culture medium was aspirated off and replaced with fresh culture medium and the cells grown at 37°C for between 4-14 days.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

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Human Genetic Signatures Pty Ltd

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